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Clinical and Experimental Study on the Tumor Cells in the Blood

by

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CHAPTER I

Clinical Study on the Tumor Cells in the Circulating Blood

INTRODUCTION

In 1869, ASHWORTH[1] reported that in the blood stream of a patient of malignant skin tumor cells of the same size and appearance as in the chief tumor were found. SCHLEIP[31], MARCUS[18], and QUENSEL[28], reported a similar case as ASHWORTH's. POOL & DUNLOP[25] were the first who examined cancer cells in the blood systematically. They collected blood specimens of about 40 cases of patients and cadavers. In the blood of 17 cases they found large cells with round or elongated hyperchromatic nuclei. Although various reports of circulating cancer cells have been published, significance of tumor cells in the blood has not yet been thoroughly understood, especially concerning their role in blood borne metastasis. In the present study, bloods of 32 cancer patients were examined.

METHOD AND MATERIAL

Thirty two patients suffering from malignant tumors were examined: twenty two patients of carcinoma of the stomach, three of ileocecal carcinoma, one of rectal carcinoma, two of cancer of the breast, one of malignant melanoma, hypernephroma, melanoblastoma, and dysgerminoma respectively.

Five ml of peripheral blood was withdrawn from the right middle cubital vein with a dried syringe and needle. When blood specimen from the draining vein of tumor was to be examined, 10ml of the blood was taken before operative manipulation of the tumors. As a control group, a healthy man, a patient of uterine myoma and a patient of granuloma of the abdominal wall were examined.

Although there are various methods[6)16)22)25)28)29)32)35)44)] (Table 1) to separate tumor cells in the blood from other blood components, the main procedure consists of centrifugation or filtration. With centrifugation method, tumor cells are found in the leucocyte layer of the sediment, because of their almost same gravity as that of leucocytes. In this study two methods of centrifugation were used; (hematocrit method and fibrinogen method).

(1) Hematocrit method recommended by UNO[44]

i) Five ml of blood was mixed with 1 ml of doubled oxalate solution in a modified BESSIS[3]' tube.

Table 1

Methods of
Isolation of Cancer Cells in the Blood

1) **"Hemolysis" method**			
(a)	Aq. dest.	(Quensel	1921)
(b)	Acetic acid	(Pool & Dunlop	1934)
(c)	Saponin	(Engell	1955)
(d)	Streptolysin 0		
	(イ) Filtration	(Malmgren	1958)
	(ロ) Centrifugation	(Long	1959)
2) **"Specific Gravity" method**			
(a)	Flotation		
	(イ) Albumin	(Roberts	1958)
	(ロ) Silicon	(Seal	1959)
(b)	Acceleration of Blood Sedimentation		
	(イ) Fibrinogen	(Sandberg	1957)
	(ロ) Hematocrit	(Uno	1958)

ii) The mixture was centrifuged at the rate of about 1500-2000 r. p. m. for ten minutes.

iii) The supernatant fluid was removed, and the white buffy coat layer was taken out with a capillary pipett.

iv) The pipetted white material was smeared on a deck slide.

(2) Fibrinogen method (SANDBERG and MOORE[32])

Sedimentation of erythrocyte in blood is accelerated by adding fibrinogen. 160mg of bovine fibrinogen (Armour pharm. Comp.) was dissolved in 4 ml of physiologic saline solution and centrifuged to remove insoluble substances. The supernatant is transfered to bovine fibrinogen solution.

i) Ten ml of blood specimen was mixed with 2mg of heparin and 4 ml of bovine fibrinogen solution. And 3-4 ml of physiologic saline solution was added in order to accelerate erythrocyte sedimentation.

ii) The mixture was kept at the room temperature for about 20 minutes.

iii) The supernatant fluid which contained nucleate cells was decanted into another tube, and then centrifuged at the rate of about 1000 r. p. m. for 5 minutes. The sediment was smeared on slide glass. These smears were examined by Giemsa (Merk), PAPANICOLAOU's and/or PAS stains.

Identification of tumor cells in the blood

Cancer cells were identified according to PAPANICOLAOU's[26] criteria of malignancy: the following points were examined.

(1) nuclear changes

i) enlargement

ii) aberrant chromatic pattern

iii) enlarged nucleoli or increase in their number

iv) mitotic activity

v) marked thickening of nuclear membrane

vi) degeneration (abnormal vacuolation etc.)
(2) cytoplasmic changes
i) changes reflected in the staining reaction
ii) cytoplasmic inclusions
iii) atypical vacuolation
(3) changes of the cell as a whole
i) enlargement
ii) aberrance in the form
iii) degeneration or necrosis

RESULTS

In most cases the number of tumor cells found in the blood specimen was usually very small. Only in one case of gastric cancer a cell clump of a diameter of about 90μ was found.

1) Cytological findings of cancer cells in the blood

The size of the tumor cells, their nuclei, and nucleoli are shown in Table 2. The

Table 2

Size of the Cancer Cells in the Blood of Various Patients

No.	Case: Kind and site of tumors	Cellular diameters in μ	Nuclear diameters in μ	Nucleolar diameters in μ
12	Cancer of the stomach	15×15	15×14	
13	〃	17×16	13×12	
16	〃	13×13	13×13	4×4
19	〃	20×15	10×8 6×6	
22	〃	15×14	15×10	3.2×2.5
23	Cancer of the coecum	15×11	13×10	
28	Cancer of the breast	13×8	13×8	
30	Hypernephroma	15×15	15×13	
32	Malignant melanoma	25×21	18×17	
Average		16×15	13×12	

average size of the cells was 16μ×15μ, and that of the nuclei was 13μ×12μ. The cancer cells had generally large nuclei and nucleoli, sometimes with aberrant chromatic pattern (Fig. 2), mitotic imitation (Fig. 1) in the nucleus. The nucleolus/nucleus ratio was more than 0.2. In some cases "atypical cells" of various forms, were observed. For example, in a case of gastric cancer, an atypical cell clump which was thought to be an agglomeration of leucocytes was observed (Fig. 8). In a patient suffering from apigmented melanoblastoma in the skull, atypical cells found in the blood were proved to be endothelial cells (Fig. 7).

2) Incidence of positive smears

The cases in which atypical cells were observed were referred to "false positive" and

were distinguished from positive cases. The positive and false positive cases are shown in Table 3.

Table 3. Number of Positive and False Positive Cases in the Blood

	No. of patients	No. of positive cases (with tumor cells)	No. of false positive cases (with atypical cells)
Cancer of the stomach	22	5	8
Cancer of the colon & rectum	4	1	0
Cancer of the breast	2	1	1
Miscell. malig.	4	2	1
Total	32	9	10

The percentage of positive cases of all the patients was 28.1%. In the blood specimens of the control group neither tumor cells nor atypical cells were observed.

Table 4 Number of Positive and False Positive Cases in the Blood of the Peripheral and Draining Veins

	Peripheral vein			draining vein		
	No. of blood samples	No. of false positive cases	No. of Positive cases	No. of blood samples	No. of false positive cases	No. of Positive cases
Cancer of the stomach	13	4	2	12	6	2
Cancer of the colon & rectum	3	2	2	4	2	1
Cancer of the breast	2	0	1			
Miscell. malig.	8	5	2			
Total	26	11	7	16	8	3

The positive cases were analysed according to the site where the specimens were taken (Table 4). Tumor cells were positive in 7 of 26 blood specimens from the peripheral vein (26.9%), and in 3 of 16 specimens from the draining vein (18.7%).

Influence of various therapies (operation, administration of anticancer agents, and radiation therapy) on appearance of tumor cells in the blood was studied successively in six cases. The blood specimens were taken from the peripheral vein two days prior to operation, from the draining vein during operation and from the peripheral vein on the 8th and 15th postoperative days (Table 5). In a patient suffering from ileocecal cancer, tumor cell was not found in the blood specimen before operation but became positive during and after a radical operation. In another patient who had metastases of hypernephroma in the lung, cervical vertebra, clavicula, and rib, malignant cells of a degenerative form became positive after a radiation therapy. In a case of gastric cancer, tumor cells had been positive in the peripheral blood before a radical operation, but it became negative on the 8th day after the operation.

In five cases which had ascites, 10 ml of the ascitic fluid was taken immediately after the abdomen was opened. The specimens were centrifuged and the precipitant was

Table 5 Influence of Various Therapies on Appearance of Tumor Cells in the Blood

Case No. Age, Sex	Name of diseases	Operative modus	Histolog. findings	Tumor cell in the blood p. b.	d.	p. a. (I)	p. a. (II)	Remarks
5 64. male	Cancer of the stomach	Subtotal gastric resection	Adenocarcinoma	−	−	−	／	Thio TEPA
23 56. male	Cancer of the colon	Hemicolectomia dex.	〃	−	+	+	／	−
25 71. male	〃	〃	〃	−	−	／	／	−
19 58. fem.	Cancer of the stomach	Subtotal gastric resection	〃	+	／	±	／	−
29 52. male	Melanoblastoma	Tumor resection	Non pigmented melanoblastoma	±	／	／	−	Mitomycin
30 43. male	Hypernephroma	Inoperable	Hypernephroma	−	／	+	−	Roentgen therapy

p. b. : Peripheral blood examined 2 days before operation
d. : Blood from draining vein during operation
p. a. (I) : Peripheral blood examined 8 days after operation or onset of roentgen therapy
p. a. (II) : Peripheral blood examined 15 days after operation or onset of roentgen therapy
+ : positive
− : negative
± : atypical cell appeared

examined microscopically. At the same time blood specimens from the draining veins were examined. In one of four patients of gastric cancer, tumor cells were found both in the blood and ascitic fluid (Table 6).

Table 6 Cancer Cells in the Blood and Ascitic Fluid

	No. of patients	No. of patients with cancer cells in the blood	No. of patients with cancer cells in the ascitic fluid
Cancer of the stomach	4	1	2
Cancer of the colon & rectum	1	0	0
Total	5	1	2

Table 7 Relationship between the Duration of Illness and Positivity of Cancer Cells in the Blood

	Ad. within 3 months		Ad. within 6 months		Ad. within 1 year		Ad. after over 1 year		Total	
	No. of Positive cases	No. of Patients	No. of Positive cases	No. of Patients	No. of Positive cases	No. of Patients	No. of Positive cases	No. of Patients	No. of Positive cases	No. of Patients
Cancer of the stomach	4	8		8	1	5		1	5	22
Cancer of the colon & rectum	1	2		1				1	1	4
Cancer of the breast					1	1		1	1	2
Miscell. malig.	1	1	1	2		1			2	4
Total	6	11	1	11	2	7		3	9	32

The relationship between duration of illness and rate of positive cases are shown in Table 7. There were 11 patients who were admitted within 3 months after their initial complaints started. Of the eight cases of gastric cancer of this group, 4 were positive.

One of two patients of cancer of colon and rectum was positive. One patient of hypernephroma was positive.

There were 11 cases which were admitted between the 3rd and 6th month after the onset of complaints. One of two patients of malignant melanoma, was positive. Of seven patients admitted between the 6th and 12th month after the onset of complaints, two were positive. None of 3 patients admitted over a year after the onset of sickness was positive.

In 18 cases of gastric cancer, relationship between the rate of positive cases and cytological findings of the primary tumors was examined. At the same time, the rate of positive cases was studied in relation to the extent of metastasis, which was determined

Table 8 Relationship between Histological Diagnosis of the Primary Tumor and Frequency of Positive and False Positive Cases (Cancer of the Stomach)

		No. of patients	No. of positive cases	No. of false positive cases
Adenocarcinoma papillotubulare	I.	8	2	4
	II.	2		
Carcinoma medullare	I.	3	2	
	II.			
Carcinoma scirrhosum	I.	2		
	II.	1	1	
Carcinoma gelatinosum	I.	1		
	II.	1		1
Total		18	5	5

I. with lymphnode metastases
II. with normal lymphnode

by usual histological examination of the removed lymphnodes. The results are shown in Table 8: in subgroup I definite lymphnode metastasis was proved and in subgroup II the lymphnodes extirpated during operation proved to be normal histologically. Of 8 patients having the diagnosis of adenocarcinoma papillotubulare two were positive and four were false positive. Of 3 patients of carcinoma medullare 2 were positive, of 3 patients of carcinoma scirrhosum 1 was positive, and of two patients of carcinoma gelatinosum one was false positive.

Table 9 Relationship between Cytological Findings (Broders) and Positive and False Positive Cases in the Blood (Cancer of the Stomach)

	No. of Patients	No. of positive cases with "cancer" cells	(%)	No. of false positive cases with "atypical" cells	(%)
Grade 1	1	0		1	
Grade 2	9	2		3	
Grade 3	6	1		1	
Grade 4	2	2		0	
Total	18	5	28	5	28

Table 9 shows the relationship between the rate of positive cases and the grade of BRODERS' classification. The patient suffering from gastric cancer of grade I of BRODERS' classification, tumor cells in the blood were not found, although atypical cells were observed. Of 9 patients of grade II, tumor cells were found in 2 cases, and atypical cells in three cases. Of 6 cases of grade III, one positive and one false positive case were recognized and all the two cases of grade IV were positive.

DISCUSSION

Concerning the incidence of tumor cells in the blood, there are many reports,[6)8)19)27)32)40)] the results in which are summarized in Table 10. There is a marked variety in

Table 10 Positive Case of the Tumor Cells in the Blood

(Peripheral Vein)

Author	No. of cases	No. of positive cases	Ratio of positive cases
Pool & Dunlop	40	17	42.5%
Engell	93	17	18.2%
Sandberg & Moore	179	93	52.0%
Roberts	92	15	16.3%
Fletcher	11	3	
Uno	339	204	60.2%
Tazaki et al.	125	35	28.0%
Yamagata	64	8	12.5%

(Draining vein)

Author	No. of cases	No. of positive cases	Ratio of positive cases
Fisher & Turnbull	25	8	32.0%
Engell	125	75	60.0%
Sandberg & Moore	109	60	55.0%
Roberts	100	21	21.0%
Tazaki et al.	52	23	44.3%

(Summarized by Author)

frequency of positivity. For example, SANDBERG & MOORE reported that 16.3% of their cases was positive, whereas SEAL reported that 64.0% was positive. The variety in the incidence of positive cases may be due to the following facts as TAZAKI[40)] stated.

(1) The amount of the tumor cells obtained in the specimens is variable according to the method employed.

(2) As there is not a common and definite criterion for identification of tumor cells, authors usually use their own criteria in identifying suspicious cells in the blood.

Two methods of centrifugation were utilized in this study. Hematocrit method was simple and inexpensive. In order to separate the nucleate cell layer from the blood specimen, the special tubes which BESSIS[3)] had used for separation of the leucocyte were modified and used. A question arose that there might be any nucleate cells in the erythro-

cyte layer obtained by hematocrit method. Three specimens were examined to answer this question. In the smears of the erythrocyte layer of the specimens only a few polynuclear cells or lymphocytes were found. The tumor cells in the blood could not be easily separated from erythrocytes, unless the specimen was centrifuged at a high rate. This resulted in physical damage of the cells. With fibrinogen method, on the other hand, such a high rate of centrifugation as that of hematocrit method was not necessary and tumor cells of less damage could be subjected to examination.

SANDBERG et al.[33] stated briefly that malignant cells could be identified because of their immature appearance, i. e. with large nuclei and nucleoli, large size, and peculiar staining properties. These characteristics make them "visual strangers" among the normal hematopoietic elements. In this study the criterion of PAPANICOLAOU[26] was adopted.

The nucleolus/nucleus ratios of cancer cells in the blood were measured in two cases. The ratios were 0.24 and 0.31, and corresponded with the result obtained by MCCARTY[21], ZADEK[48], and ENGELL[6]. The atypical cells were considered to be non malignant cells derived from the hematopoietic, endothelial, or unknown origin (Fig. 6-12). For example, the atypical cells in Fig. 7 were thought to be endothelial cells.

The fact that tumor cells are floating in the circulating blood means cancerous involvement of the blood and lymph vessel and release of the invading tumor cells into the blood stream. In 1865, THIERSCH[41] observed histological findings of involvement of lymphatic and blood vessels by tumor cells. GOLDMANN[9], in 1897, pointed out that venous invasion was often demonstrable even at an early stage. In our studies, relationship between the grade of cancerous involvement of the vessels and the rate of positive cases was not examined systematically. BROWN and WARREN has reported that cancerous involvement of the veins and blood borne metastasis are in proportionate to the degree of differentiation of tumor cells. As ENGELL studied, the positivity of tumor cells in the blood was higher in grade III and IV than in grade I and II of BRODERS'[4] classification. Table 9 seems to indicate that the more undifferentiated the tumors, the higher the rate of positive cases.

The fact that higher rate of the positive cases was observed in patients who had admitted within shorter period after onset of their sickness might indicate that the more rapid the tumor growth is the higher the rate of positive cases.

Whether cancer cells in the blood can develop metastatic foci or not depends not only on their validity but also on the site, "the soil", where they are arrested. As MOORE et al.[20] pointed out, the majority of the tumor cells released into blood stream is supposed to fail to survive or establish foci of metastases. It is well known[20][49][50] that tumor cells easily pass through the capillary bed by their ameboid movement and deformation. The data that the positivity was higher in the blood specimens from the peripheral veins than in those from the draining veins is inconsistent with other author's[6][40] results. The findings suggest that the floating tumor cells in the blood can pass through the capillary bed of the lung and liver.

In some cases which were negative before operation or radiation therapy, tumor cells in the blood became positive after the treatments. This must be due to the influence of physical operative manipulation or radiation therapy on release of the tumor cells into circulating blood. KNOX[13], ROBERTS et al.[29], and IWASAKI[11] reported mechanical release

of the tumor cells into the blood stream caused by massage of the tumor and by operative or diagnostic procedures.

ENGELL[7] reported that in the patients who survived 5 to 9 years after operations, the tumor cells in the blood became negative in long term follow up, and suggested that the positivity of tumor cells in the blood did not necessarily coincide with incurability. On the contrary, UNO[44] concluded that appearance of cancer cells in the blood was a sign of poor prognosis.

Anyway, ligation of the feeding artery as well as the draining vein at an early stage of operative procedure is said to be important to prevent blood borne metastasis. The tumor cells in the ascites, on the other hand, may be the source of dissemination of the cancer in the free abdominal cavity. Of five cases with ascites, tumor cells were observed in the ascitic fluid in two cases, one of which was also positive in regard to the cancer cells in the blood specimen. Judging from these results, it seems rational that the anticancer agents should be administered systematically as well as locally in order to prevent both blood borne and disseminated metastasis.

CHAPTER II

Experimental Studies on Tumor Cells in the Blood

INTRODUCTION

HANS LOEWENTHAL[17], TAKAHASHI[12], SASAKI[36], and NISHI[24] had studied on the fate of intravenously injected experimental ascitic tumor cells. Such a study by use of an ascitic form of tumor, however, is supposed to be inadequate to elucidate the actual clinical situation of most patients suffering from solid tumors.

In this study experimental solid tumors were used in order to investigate the fate of tumor cells in the blood and influeuces of anticancer agents on blood borne metastasis.

PRELIMINARY EXPERIMENTS

§ 1. Artificial lung metastasis of MC/5 tumor

MC/5 is a mammary cancer which grew up spontaneously in mice of dd strain in 1959 and has been successively transferred by MIYAWAKI[33]. Cell suspension of this tumor was injected intravenously into homologous mice. The various organs of the mice which were sacrificed a few days after the inoculation were examined histologically.

(1) Animal

Forty to sixty day old male mice of dd strain, weighing about 20gm, were fed with ordinary mice food (the Oriental Yeast Corp. Tokyo), and supplied with water ad libitum. Eight mice were set as a group.

(2) Method of preparing coarse cell suspension

Tumors were extirpated aseptically on the 10th to 12th day after inoculation. After discarding the central part of tumors, the tumor tissue was minced with scissors and roughly homogenized with POTTER's homogeneizer, adding about three volumes of physiologic saline.

After few minutes, whitish turbid fluid was removed from the middle layer of the homogenate and used as coarse cell suspension. 0.2 ml of the coarse cell suspension was injected into the tail vein of dd mice.

(3) Results

Twenty one of sixty three mice succumbed probably from embolism immediately after intravenous injection of the cell suspension. Forty two survived mice were sacrificed on the 26th day after the intravenous injection, and the histological examination was done on the brain, heart, lung, liver, spleen, and retroperitoneal lymphnodes. In fifteen mice, macroscopical lung metastasis was found. Histologically the metastasized tumor was carcinoma simplex and invaded the alveolar structure of the lung (Fig. 13). In other organs, histological findings did not show any sign of metastasis formation. Intravenous injections of the cell suspension caused immediate death of many mice, and gave low rate of lung metastasis.

The results suggest that MC/5 is not suitable for the study on the fate of intravenously injected tumor cells, and the evaluation of the effect of anticancer agents against blood borne metastasis.

§ 2. Artificial lung metastasis of Bashford carcinoma 63

Bashford carcinoma 63 supplied from the Shionogi Research Institute, Osaka, was used to study the relationship between the number of tumor cells which were contained in an inoculum (cell suspension) and its metastasizability. In this experiment the cell suspension was filtered through a cytosieve in order to remove tissue fragments which might cause embolism when injected intravenously. Nembutal (Abbott Lab.) anesthesia was given to the animals just before the inoculation of the filtrate.

(1) Method

Male mice, 40 to 60 days old, weighing about 20gm were used. Ten mice were set as one group. The coarse cell suspension prepared as previously described was filtered with two cytosieves, the sizes of which were 100μ to 120μ and 44μ to 32μ.

The cell suspension (Fig. 14) obtained in this way of filtration contained mostly single cells and a few cell clumps. Cytoplasm was apt to be destroyed by the homogenizer, while most caryoplasm was left free from mechanical damage. Number of tumor cells in the cell suspension was measured by melangeur (hemocytometer for the white blood cell), and adjusted in such a way that one single dosis of inoculation, not more than 0.2 ml in all cases, contained about one million cells. All mice were anesthetized with 50mg/Kg of nembutal. Then cell suspension was injected very slowly into the tail vein. The number of cells in the suspension was variable in each group as shown in Table 11. Mice were examined until the death from the tumor. Some of them were sacrificed on the 60th day after the inoculation to examine metastasis.

(2) Results

Some mice succumbed immediately after injection of tumor cell suspension as those seen in MC/5. These mice were excluded from the result. Most of the mice which were died from the tumor had several or multiple metastatic nodules in the lung as seen in Fig. 15.

One of six mice which were injected intravenously with 37,000 cells had lung

Table 11 Relationship between Number of Intravenously Injected Bashford Carcinoma Cell and Artificial Lung Metastasis Formation

Cell counts	No. of mice	No. of mice died from lung metastasis	Ratio of tumor death (%)	Average of survival time (days)
37×10^4	6	1	16%	
60×10^4	6	2	33.3%	23
62×10^4	7	4	57.1%	24.5
68×10^4	6	4	66.7%	42.5
75×10^4	7	5	71.4%	21.4
82×10^4	9	8	88.9%	26
86×10^4	7	7	100%	24.4
92×10^4	8	7	87.5%	19.7
100×10^4	9	8	88.9%	20.5
114×10^4	10	4	40%	19.3
118×10^4	5	4	80%	22.5

metastasis; two of six mice injected with 60,000 cells died from lung metastasis, as shown in Table 11. As the number of injected cell increased, the rate of lung metastasis increased. Intravenous injection with 850,000 to 1,000,000 cells produced lung metastasis in about 90 per cent of all cases. The mice with lung metastasis died at almost the same period after inoculation independently on the number of the inoculated cells. And their average survival time was 24.4 days.

(3) Histological findings

Histological examination revealed that lung metastasis was the most frequent and there was not any favourite site of metastasis in the lung. In some of the mice with lung metastasis, adhesion between the tumor and the parietal pleura and hemothorax were observed.

In the heart and the tail of some cases also tumor growth was observed, but not in any other organs. The tumor growth in the heart located in the right auricle as shown in Figs. 23 and 24. The tumor cells invaded into the myocardial tissue. In the tail, the tumor infiltrated into the soft tissue around the tail vessel.

EXPERIMENTS ON THE FATE OF INTRAVENOUSLY INJECTED CANCER CELLS

Cell suspensions were made from Bashford tumor in the same way as that of the preliminary experiment. The cell suspension was injected intravenously to mice. The blood specimen taken from the peripheral vein and the heart of the mice (donors) were examined to know how long the injected cells stay in the blood, then the intracardiac blood of the mice were injected to other mice (recipients) in order to know metastasizability of the tumor cells which might be contained in the intracardiac blood of the donor mice.

§ 1. Disappearance of intravenously injected Bashford carcinoma cells from peripheral circulation

(1) Method

Cell suspension of about 110×10^4 of Bashford carcinoma cells were injected intravenously into the tail vein of 8 mice. At a various period after the injection about 0.02 ml of blood specimen was taken from the tail vein on the opposite side of each mouse. The

specimen was smeared on a slide glass, and examined microscopically for exsistence of the tumor cells in the blood.

(2) Results

Table 12 Disappearance of the Injected Cancer Cells in the Peripheral Blood

Time after intravenous injection of cell suspension	5	10	20	30	45	60	90 m.	2	6	12	24 h.	2	4	7	9	11	13 d.
No. 1	+		+		+		−		−		−		−		−		−
No. 2	+		−		−		−		−		−		−		−		−
No. 3	+		+		+		−		−		−		−		−		−
No. 4	+		+		+		−		−		−		−		−		−
No. 5		+		+		+		−		−		−		−		−	
No. 6		+		−		−		−		−		−		−		−	
No. 7		+		−		+		−		−		−		−		−	
No. 8		+		−		+		+*		−		−		−		−	

\+ tumor cells detectable in the blood
− non detectable
+* abnormal cells detectable

The results are shown in Table 12. The tumor cells were detectable within 10 minutes after intravenous injection of tumor cells. The tumor cells in the blood underwent degenerative changes: the nuclear membranes were destroyed, and the networks were damaged, showing caryolysis (Fig. 16). The later the specimen were taken, the more severe these changes were. Almost one hour after the injection, most cancer cells seemed to perish from the circulating blood.

§ 2. Growth of tumor cells arrested in the lung

(1) Method

The experiment was planned to observe the growth of tumor cells arrested in the capillary beds of the lung. At a various stage from the 2nd hour to the 13th day after injecting the tumor cells intravenously in the same way as that of § 1, the mice were sacrificed, and serial sections were made from the right lung for histological examination. The sections were prepared by formalin fixation and hematoxylin eosin stain.

The mice which were killed at the 2nd hour following the injection showed tumor cells, in some cases resembling necrotic cells, filling the majority of small vessels in the lung. Around the vessels filled with tumor cells, polymorphonuclear leucocytes and erythrocytes infiltrated into alveolar tissue (Fig. 17). In the specimen removed on the 6th hour, cancer cells were found outside of the vessel wall, around which cell infiltration and hemorrhage were also observed (Fig. 18). On the 12th hour after the intravenous injection, tumor cells were proliferating in the perivascular spaces (Fig. 19). On the 24th hour, alveolar tissue around peribronchial lymphnodes showed congestion and swelling. On the 7th day, the size of metastatic foci became larger, and findings of destruction of the alveolar tissue were observed (Figs. 20, 21, 22).

§ 3. Disappearance of intravenously injected Bashford carcinoma cell from intracardiac blood

In this experiment tumor cells in the intracardiac blood of mice which had been inoculated with the tumor cells in the same way as in the experiment of § 1 were examined.

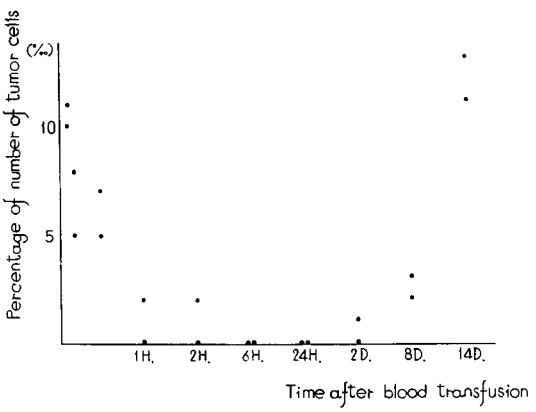
(1) Method

About 0.02 ml of the blood specimen taken from each mouse which had been inoculated with the tumor cells intravenously was examined for existence of tumor cells in the blood by the smear method, and the number of tumor cells in the intracardiac blood was counted.

(2) Results

The number of tumor cells in the intracardiac blood decreased in accordance with time lapse after the inoculation, and on the 6th and 24th hour after the inoculation no cancer cells were observed in the intracardiac blood. On the 8th day after the intravenous inoculation, however, tumor cells appeared again and their number increased thereafter, as

Fig. 28



shown in Fig. 28. Bashford carcinoma cells found in the intracardiac blood were in the form of single cells or cell clumps of the several cells, as shown in Figs. 25, 26.

§ 4. Metastasizability of floating cancer cells in intracardiac blood of mice

By transfusing intracardiac blood specimen which might contain tumor cells to secondary recipient mice, metastasizability of the tumor cells was examined.

(1) Method

Six mice of dd strain were set up as a group. Donor mice were inoculated intravenously with about 1,000,000 of Bashford carcinoma cells.

Five, 10, 30 minutes, 1, 2, 6, 24 hours, 2, 8, and 14 days after intravenous inoculation of the carcinoma cell suspension, blood of the donor mice was withdrawn, and transfused to the groups of recipient mice. Some donor mice expired suddenly after intravenous inoculation of the carcinoma cell suspension and some recipient mice died after the transfusion of intracardiac blood taken from donor mice.

(2) Results

All the survived mice were sacrificed on the 60th day after the transfusion of the

intracardiac blood.

As shown in Table 13, one of four mice which were transfused with the intracardiac

Table 13 Metastasis Formation Produced in the Secondary Recipient Mice by the Transfusion of the Intracardiac Blood Which Was Taken from the Donor Mice at Various Stages after Intravenous Injection of Bashford Carcinoma Cell Suspension

*Time	No. of Mice	Dead Immed.	No. of Surv. Mice	Metastasis		Metastasis ratio
				Yes	No	
5 M.	9	5	1	0	4	0
10 M.	4	0	4	1	3	25
30 M.	6	5	1	0	1	0
1 H.	6	1	5	0	5	0
2 H.	5	1	4	0	4	0
6 H.	6	3	3	0	3	0
24 H.	5	0	5	1	4	20
2 D.	4	2	2	0	2	0
8 D.	6	2	4	2	2	50
14 D.	12	10	2	1	1	50

*Time: Time lapse between the intravenous injection of the cell suspension and removal of intracardiac blood

blood taken 10 minutes after intravenous inoculation of the coarse cell suspension had lung metastasis (Fig. 27). One of five mice transfused 24 hours after the inoculation, two of four mice transfused eight days after the inoculation, and one of two mice transfused fourteen days after the inoculation had also artificial lung metastasis.

EFFECTS OF ANTICANCER AGENTS ON ARTIFICIAL LUNG METASTASIS OF BASHFORD CARCINOMA 63

Although many reports have been published in the field of anticancer chemotherapy, there are only a few reports[2)10)14)15)] concerning the effect of anticancer agents on blood borne metastasis. In this study, at first, effects of various anticancer agents on tumor growth and survival time of mice which were inoculated subcutaneously with Bashford carcinoma 63 were examined. Then chemotherapeutic effects of the drugs on artificially induced lung metastasis of Bashford carcinoma 63 in mice were studied.

Various anticancer agents, Triethylene thiophosphoramide, Mitomycin C, and Toyomycin (Chromomycin A_3) were used. Triethylene thiophosphoramide is abbreviated as thio TEPA, Mitomycin C as MMC., and Toyomycin as Chr. A_3.

§ 1. Effect of anticancer agents on subcutaneous tumor of Bashford carcinoma 63

(1) Method

Mice were divided into 4 groups and eight mice were set as a group. A tumor of Bashford carcinoma, 10 days after inoculation, was aseptically extirpated. A piece of tumor tissue was taken from the marginal portion of the tumor and was inoculated subcutaneously to normal mice with a trocar. One fifth of LD50 of various anticancer agents were administered to the mice intraperitoneally three times every other day, starting on the 8th day after the inoculation.

Single dosis of anticancer agents given to each group is as follows:

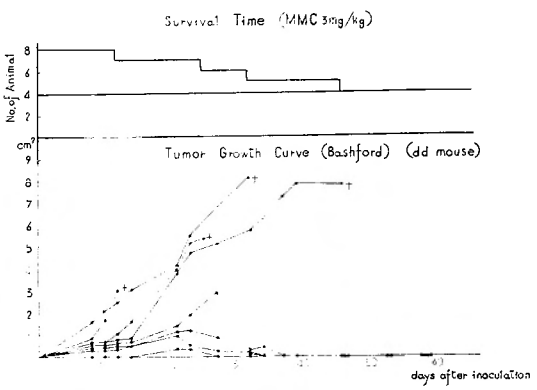
MMC	group :	1mg/kg of MMC
Chr. A_3	group :	400mcg/kg of Chr. A_3
thio TEPA	group :	3.5mg/kg of thio TEPA
Control group :		0.2ml/mouse of physiologic saline

The size of tumors was calculated by measuring the maximal length and its vertical length of tumors. The mice were examined till the 60th day after the subcutaneous inoculation of the tumor tissue.

(2) Results

a) MMC group

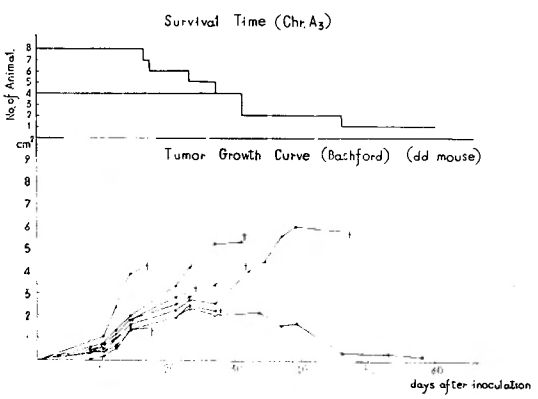
Fig. 29



Tumor growth in MMC group is shown in Fig. 29. Tumor regression was observed in one mouse. In three of nine mice, the tumor reduced its size from the 22nd day after the inoculation, and became unpalpable on the 38th day. Fifty percent survival time was 46 days in this group.

b) Chr. A_3 group

Fig. 30



The tumor in this group (in Fig. 30) grew continuously in all cases excepting one case which showed reduction of tumor size on the 35th day after the inoculation. Mice in this group started to die on the 4th day after the administration of Chr. A_3, possibly due to toxicity of Chr. A_3. Fifty percent survival time in this group was 28 days.

c) thio TEPA group

Fig. 31

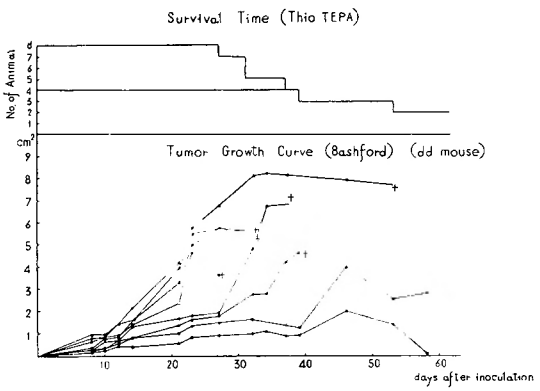
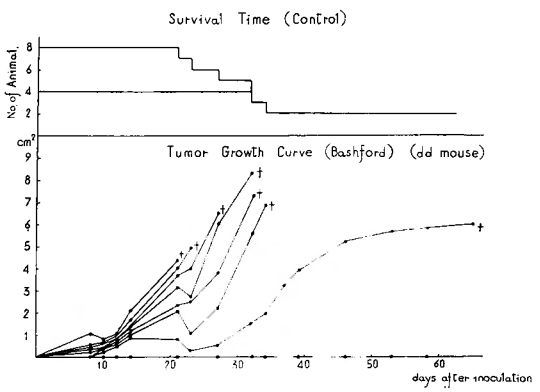


Fig. 31 showed the survival time and tumor growth curve of this group. In two mice, tumor growth was suppressed, while the other mice started to die on the 26th day after the inoculation. Fifty percent survival time was 36 days.

d) Control group

Fig. 32



In one mouse no tumor was palpable on the 8th day after inoculation. Six of 7 mice succumbed between the 20th and the 35th day, and one of 7 mice had gradual growth of tumor, and finally expired on the 70th day (Fig. 32). Fifty percent survival time was 32 days.

§ 2. Chemotherapeutic effects on artificial lung metastasis of Bashford carcinoma 63

(A) Effect of MMC

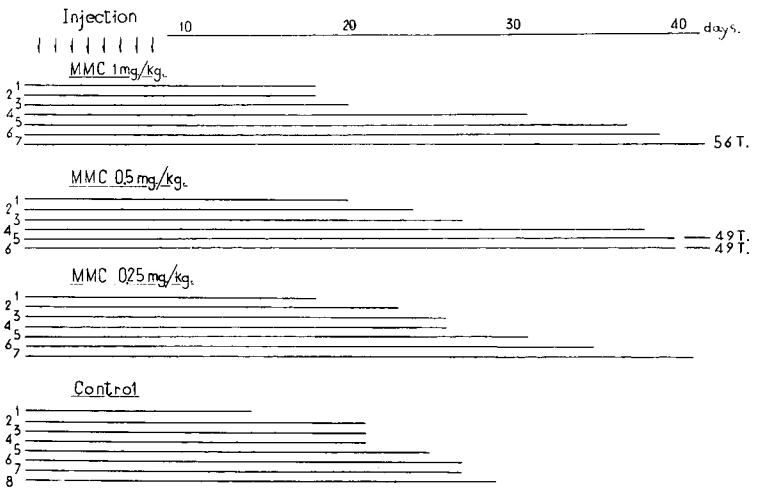
(1) Method

Eight mice of dd strain were set as a group. Each mouse was injected with a suspension of about one million cells of Bashford carcinoma into the tail vein. Intraperitoneal MMC injection was given once a day for successive 8 days, starting 24 hours after the inoculation of the tumor cells. The mice were divided into 4 groups.

The daily dosis of the drugs were as follows :

Group I :	1mg/kg of MMC
Group II :	0.5mg/kg of MMC
Group III :	0.25mg/kg of MMC
Control group :	0.2ml/mouse of physiologic saline

Fig. 33



Survival time of the mice with lung metastasis which are injected intravenously with Bashford carcinoma cell suspension and treated with MMC

(2) Results

Survival time of each mouse is shown in Fig. 33. Average of survival time of group I was 31.3 days, that of group II 34.5 days, that of group III 28.6 days, while the average of control group was 26.0 days. The average survival times of the groups treated with MMC were larger than that of the control group.

In MMC treated mice, apparent correlation between the survival times and dosis of MMC was not observed.

(B) Effect of Chr. A_3 and thio TEPA on artificial lung metastasis

(1) Method

Mice of dd strain were divided into 4 groups. Eight mice were set as a group. The mice were injected with about 100×10^4 tumor cells into the tail vein in order to make lung metastasis. Twenty four hours after the inoculation of tumor cells administration of

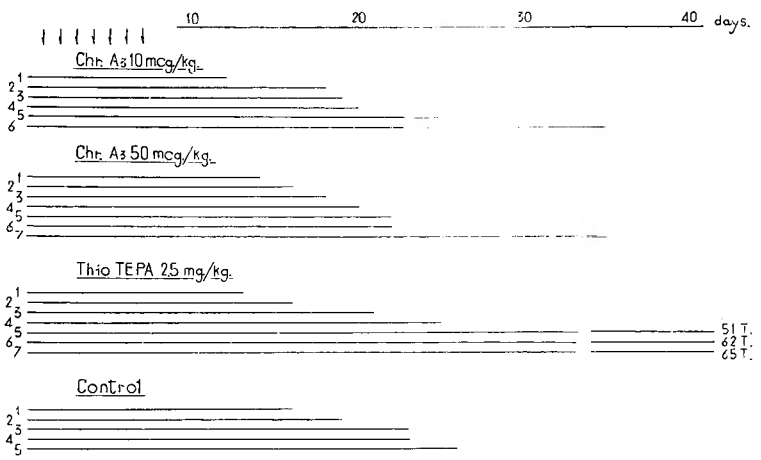
anticancer agents was started. The agents were given once a day for 7 successive days.

The single dosis of the agents was as follows :

Group	I :	10mcg/kg of Chr. A_3
Group	II :	50mcg/kg of Chr. A_3
Group	III :	2.5mg/kg of thio TEPA
Control group :		0.2ml/mouse of saline

(2) Results

Fig. 34



Survival time of the mice with lung metastasis which are injected intravenously with Bashford carcinoma cell suspension and treated with Chr.A_3 and thio-TEPA

Survival time of each mouse is shown in Fig. 34. Average survival time of Group I was 21.5 days, that of group II 21.0 days, that of group III 31.6 days, while the average of control group was 21.4 days. The average survival times of group I and II were no more than that of the control group, whereas that of group III was apparently larger.

DISCUSSION

Free floating tumor cells are found in the peripheral circulating blood of some patients suffering from malignant tumors. In some tumor bearing animals, tumor cells are also reported to be proved in their circulating blood[30)47)]. In this experiment Bashford carcinoma was used, and its suspension was injected into the tail vein of dd strain mice in order to study blood borne metastasis.

The study was planned to clarify whether Bashford carcinoma cells in the form of floating state could make metastatic foci in the lung of the secondary recipient mice or not, and to examine the appearance of the cancer cells in the peripheral and intracardiac blood. SATO[38)] pointed out that most of experimental studies of this kind are far from the natural situation of metastasis formation. But some informations which might be useful

to prevent blood borne metastasis could be obtained from our results.

The data of our experiment showed that the rate of metastasis formation increased proportionally to the number of injected cancer cells. The fact that the number of artificial lung metastasis is proportional to the number of injected cells was also reported by ZEIDMAN[51], COMAN[5] and RHOMSDAHL[30]. WATANABE[45] pointed out that the blood borne metastasis could not be caused by a single cell of bronchogenic carcinoma which had grown spontaneously in CBA mouse, but by a cell clump which was supposed not to be affected by "adverse action" in the blood. The coarse cell suspension of our experiments consisted almost of single cells, which made lung metastases in proportion to their number.

Peripheral blood of mice was examined successively after intravenous inoculation of the coarse cell suspension. Although most of the cancer cells were arrested at some places whithin the recipient body, some cancer cells were found still floating in the circulating blood within one hour after the injection. The fact that the cancer cells in the blood undergo degenerative changes suggests that hosts may have some "defence mechanism" which inactivates the cancer cells in the blood. SCHMIDT[39] reported 40 cases of malignant tumors in which cancer cells were enclosed in thrombus of lung capillary and no macroscopic metastatic foci were observed. He thought that most cancer cells in the blood may be degenerated and perished after all, and that cancer cells in the blood stream have to perform an organic connection with the wall of blood vessels in order to grow at the site where the cancer cells are arrested. In 1915, IWASAKI[12] showed that destruction of floating tumor cells not only of human neoplasms but also of animal tumors occurred in consequence of thrombus organization. The histological findings of our experiment revealed that the cancer cells which had been arrested in the lung capillary permeated the capillary wall into the perivascular space so soon as 6 hours after the intravenous inoculation, and began to proliferate in 12 hours. This time lapse which was necessary for the cancerous proliferation in the perivascular space was much less than that which was presented by WARREN & GATE[46] in the experiments of Walker carcinosarcoma 256.

After intravenous inoculation of the Bashford carcinoma cell, the number of floating cancer cells in the intracardiac blood decreased with time lapse as those in the peripheral blood. On the 8th and 14th day after intravenous inoculation of Bashford carcinoma cell suspension, however, they appeared in the intracardiac blood again and their number increased rapidly thereafter. The transfusion of the intracardiac blood withdrawn from the donor mice at that time resulted in producing artificial lung metastasis in the recipient mice. In these cases, the cancer cells in the intracardiac blood which caused lung metastasis in the recipient mice seemed to originate from metastatic foci in the lung of the donor mice. It can not be denied that some cancer cells in the blood actually cause blood borne metastasis of experimental tumors. Therefore, control of cancer cells which are floating in the blood is thought to be important to prevent metastasis. Using Yoshida sarcoma cells, IWASAKI[11] has studied the fate of intravenously injected tumor cells. According to HONJO & TAGASHIRA[43], Yoshida sarcoma is apt to produce leucemic or leucemoid changes in the blood of the animals when injected intravenously ; the number of leucocytes increases in accompany with the appearance of the tumor cells in the blood. In our experiments, the cancer cells injected intravenously underwent degenerative changes and

perished gradually, and any leucemic or leucemoid reaction was not observed in the peripheral blood.

Chemotherapeutic experiments of Bashford carcinoma has been reported by only a few authors[37]. In our experiment, the drugs which were effective for the treatment of subcutaneously inoculated tumors were also effective against artificial lung metastasis. The survival time of our animals was prolonged with an administration of MMC and thio-TEPA. The results of treatment with Chr. A_3 were not so good.

SUMMARY AND CONCLUSION

A) 32 patients suffering from malignant tumors were studied on existence of tumor cells in the blood obtained from the peripheral and/or the draining vein.

1) In 9 of the 32 patients (28.1%), tumor cells were positive in the blood, and in 10 patients atypical cells were positive. In the blood specimens from the peripheral vein, 7 of the 26 cases (23%) were positive, and in the blood specimens from the draining vein, 3 cases of 16 (18.8%) were positive.

2) The shorter was the course of illness before the admission, the higher was the incidence of positivity in the blood.

3) In the patients suffering from cancer of the stomach, incidence of positivity were proportional to the degree of BRODERS' grade and tended to become higher in the carcinoma medullare.

B) Artificial lung metastasis introduced by intravenous inoculation of Bashford carcinoma cell suspension was examined, especially in regard to chemotherapeutic effects.

The results were as follows :

1) The metastasizability of the Bashford carcinoma 63 to the lung of the mice was proportional to the number of intravenously inoculated tumor cells.

2) Within 1 hour after the intravenous inoculation, the tumor cells were still detectable in the peripheral blood.

3) The tumor cells permeated the capillary wall into the perivascular space so soon as 6 hours after the intravenous inoculation, and about 12 hours after the inoculation, the tumor cells started to proliferate in the perivascular space.

4) The artificial lung metastases were produced in the secondary recipient mice by transfusion of the intracardiac blood which was taken from the donor mice 8 or 14 days after intravenous inoculation of the Bashford carcinoma cell suspension.

5) Chemotherapy for artificial lung metastases which were introduced by inoculating cell suspension intravenously was performed. Anticancer agents which were effective to suppress growth of subcutaneously inoculated tumors were also effective against artificial lung metastases.

I would like to express my deepest gratitude to Assistant Professor Dr. IKUZO YOKOYAMA for his cordial guidance throughout this experiment.

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(* Written in Japanese)

和文抄録

血中腫瘍細胞についての臨床的実験的研究

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悪性腫瘍の治療成績の向上を妨げる最大の因子の一つに遠隔転移の問題がある．

近年流血中に出現する腫瘍細胞に関する研究が行なわれ，血行性転移に於ける意義が注目されてきたが，いまだ充分明らかにされていない．

本研究に於いては，まず臨床例につき悪性腫瘍患者の正中肘静脈，並びに術中の腫瘍領域静脈血中の腫瘍細胞を検索し，手術・制癌剤投与等の処置並びに腹水内腫瘍細胞の有無，腫瘍の病理組織学的所見等との関係を検索した．次に実験的に，MC 5・Bashford Carcinoma 63 を使用して固型癌による人工的肺転移を作成して，尾静脈並びに心内血中腫瘍細胞の消長，肺臓における転移形成の形態学的変化，心内血中腫瘍細胞の転移形成能の有無，並びに転移性腫瘍に対する制癌剤の影響を検討して次の結果を得た．

Ⅰ．臨床例の血中腫瘍細胞を採集するためにはヘマトクリット法，並びにフィブリノーゲン法を用いた．無選択に選んだ悪性腫瘍患者32例中9例(28.1%)に流血中腫瘍細胞の出現を認め，10例に atypical cell の出現を認めた．主訴発現より入院迄に要した期間が短いもの程血中腫瘍細胞陽性率が高く，腫瘍の組織像では腫瘍細胞の分化度の低いもの程血中腫瘍細胞の陽性率が高いという結果を得た．手術並びに化学療法，レントゲン療法施行と血中腫瘍細胞出現との間には検索した範囲内では特に明瞭な関連は認められなかつた．術前陰性であつた廻盲癌症例で術中並びに根治術術後8日に腫瘍細胞の血中出現を認めた例もある．腹水中の腫瘍細胞陽性率との関係については，検索した癌性腹水患者5例中2例に腹水中腫瘍細胞が陽性で，その中の1例にのみ血中腫瘍細胞が陽性であつた．

Ⅱ．実験腫瘍 Bashford carcinoma 63 の粗細胞液をつくり，マウスの尾静脈より注入したが，細胞数を多くすると，肺に転移腫瘍を生ずるマウスの数が増し，且腫瘍死するマウスの数が接種細胞数に比例して増加した．血中に接種した腫瘍細胞は血中で，漸次変性をうけ約1時間を経過すると尾静脈血中には最早認めがたくなつた．心内血中の癌細胞は尾静脈血中のものと同様漸減し接種後6時間24時間の標本には認められなくなつた．しかるに接種後8日頃から再び出現し，その後急激に増加の傾向を示した．粗細胞液を尾静脈内に接種すると腫瘍細胞は肺組織に定着し6時間ですでに血管外に出て，約12時間で血管外性の腫瘍性増殖を開始した．尚上記の如く粗細胞液を接種後経時的に心内血を採血し，これを他群のマウスの尾静脈より接種する事により心内血中腫瘍細胞の転移性の有無を検索した．粗細胞液接種後，10分の心内血は他のマウスに輸血した場合4匹中1匹同様の接種後24時間の心内血は5匹中1匹，同じく8日の心内血は4匹中2匹又14日後のものは2匹中1匹に夫々著明な肺転移を形成し，いづれも腫瘍死した．此の事実と上記粗細胞液接種後の肺組織像の変化とを考え合せれば，静脈内に接種された粗細胞液中の Bashford 癌細胞はまず肺転移をつくり，接種後8日後にはこの転移巣から転移形成能を有する癌細胞が出現してくる事が分つた．Bashford 癌の粗細胞浮遊液接種による人工的肺転移に対する制癌実験では，主腫瘍に対する制癌効果と同じく，マイトマイシン・テスパミンに若干の延命効果を認め，血行性転移のもととなる血中腫瘍細胞に対する制癌剤投与の意義がうかがわれた．

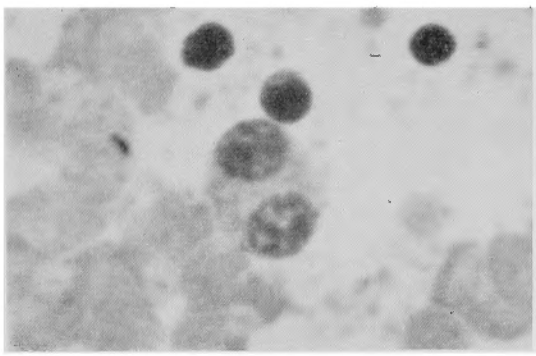
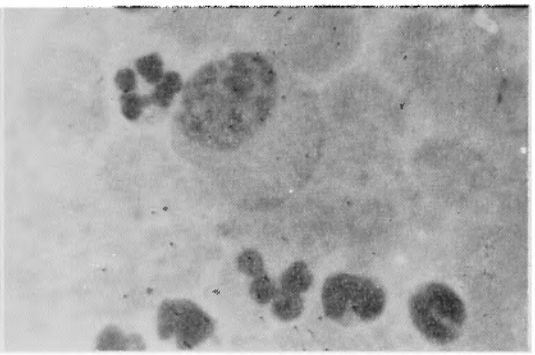


Fig. 1



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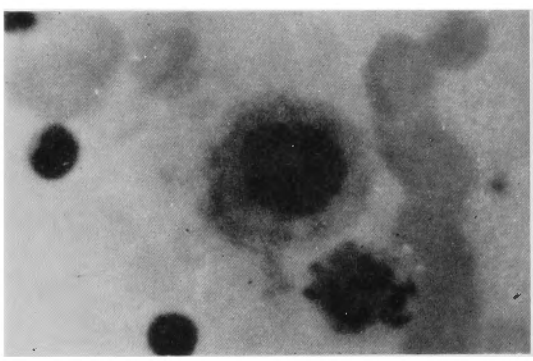

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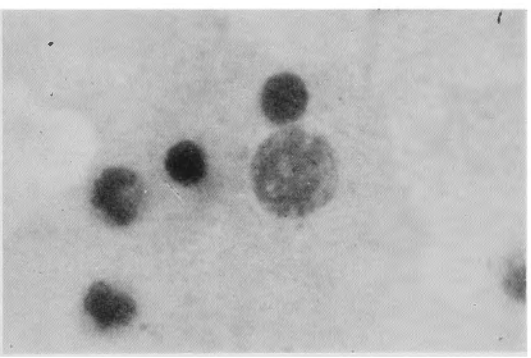

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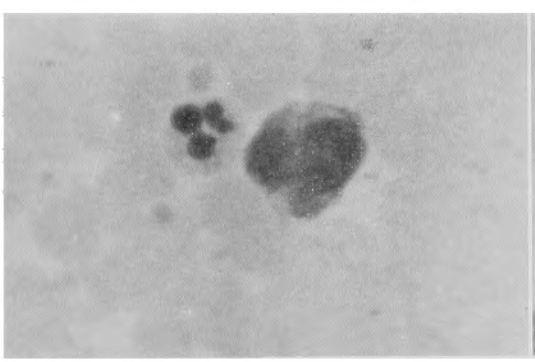

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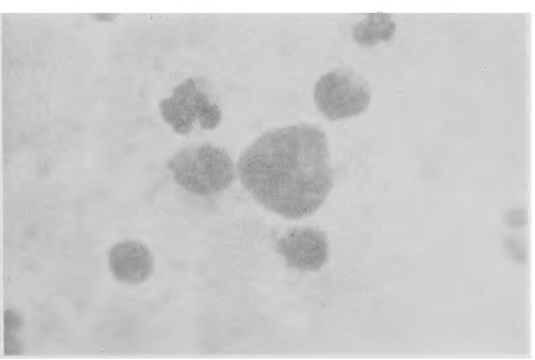

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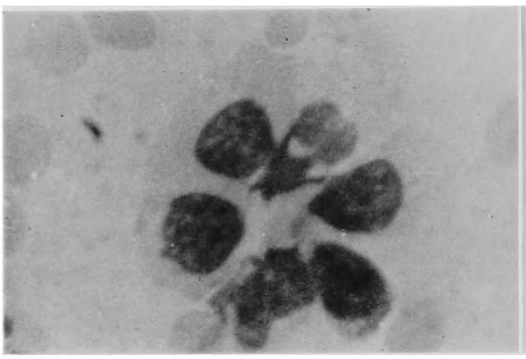

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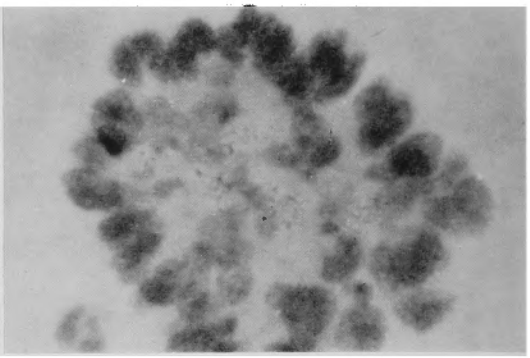

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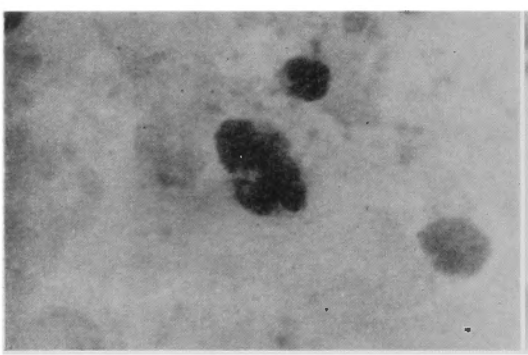

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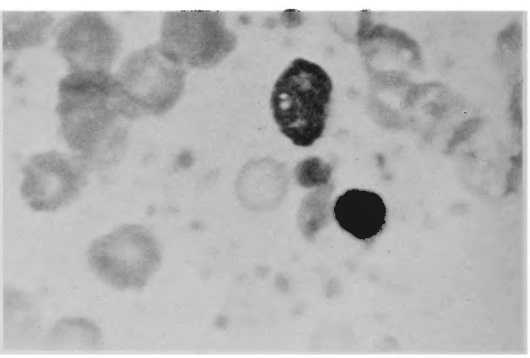

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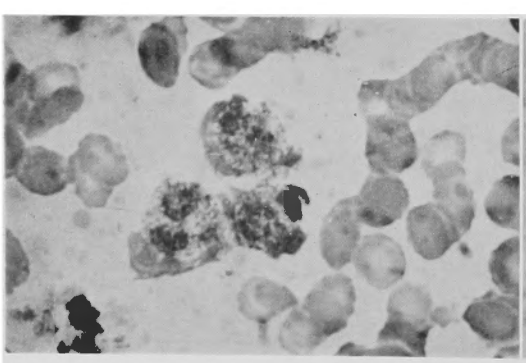

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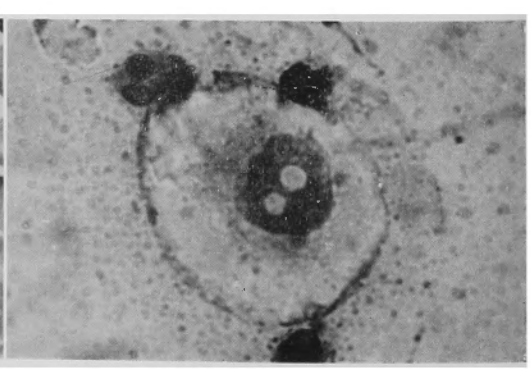

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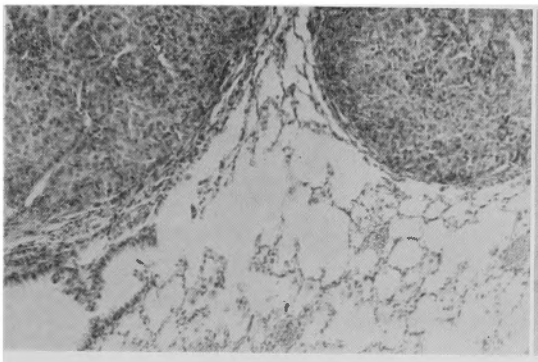

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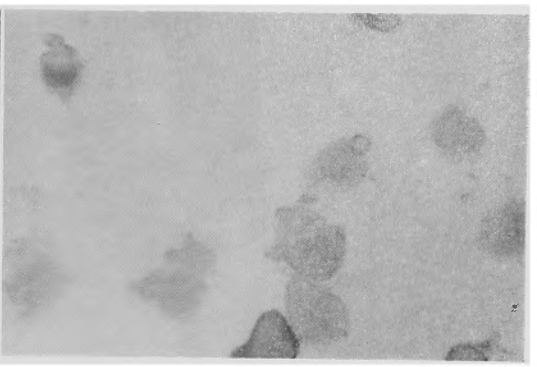

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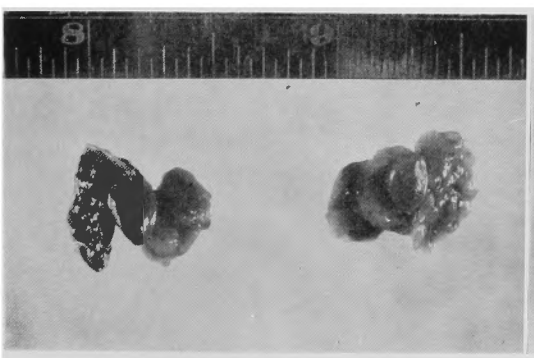

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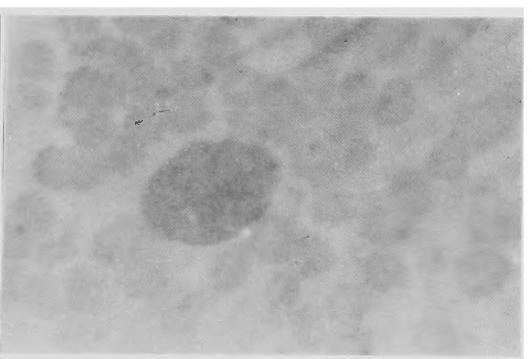

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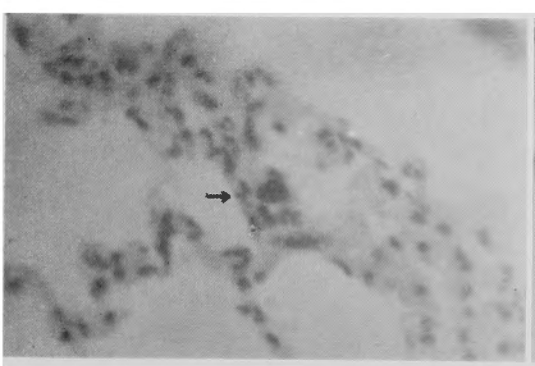

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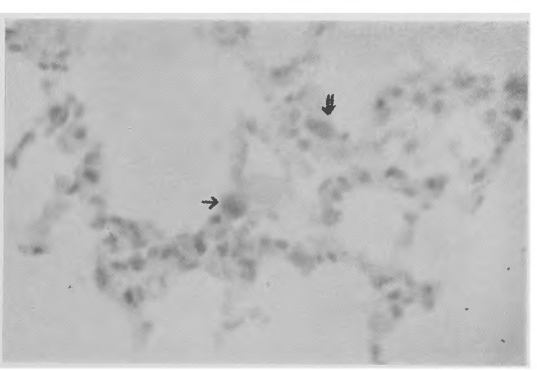

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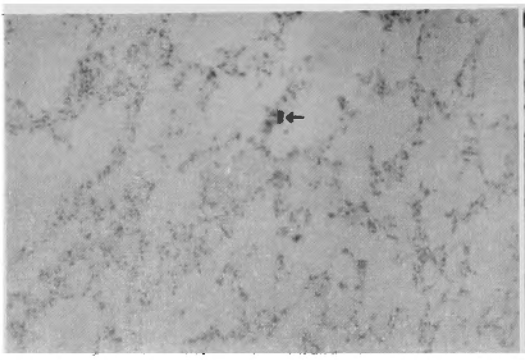

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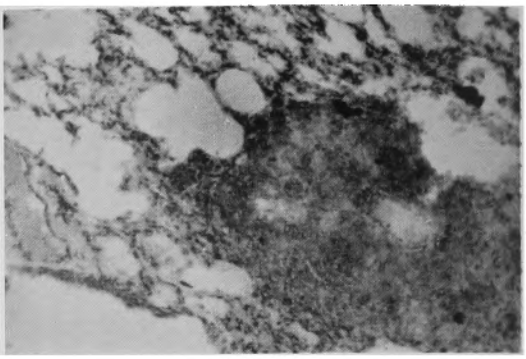

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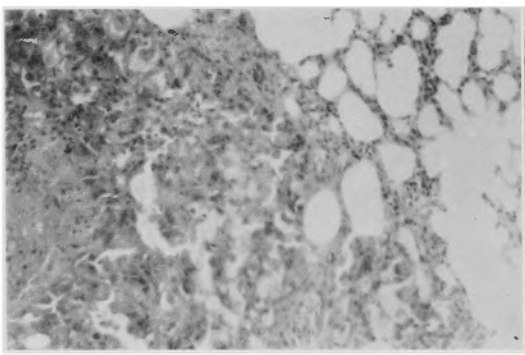

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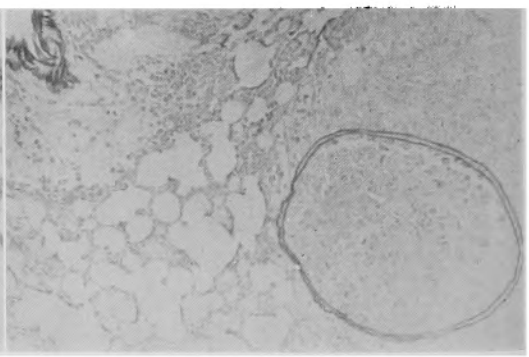

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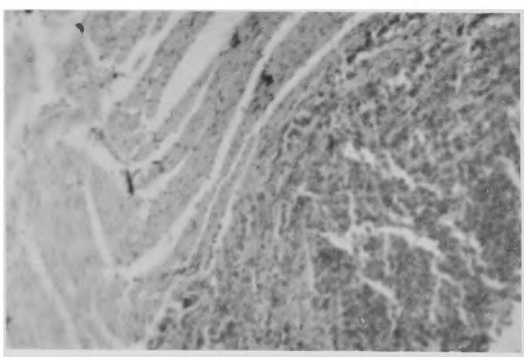

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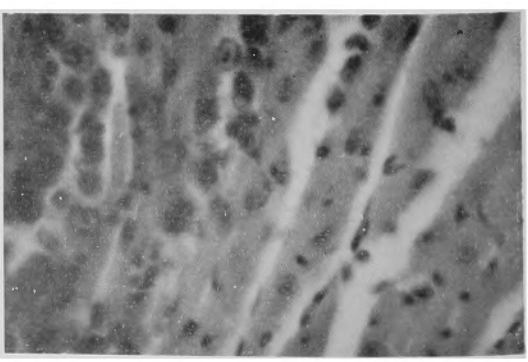

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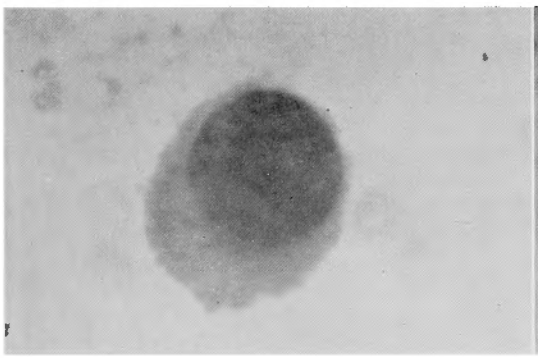

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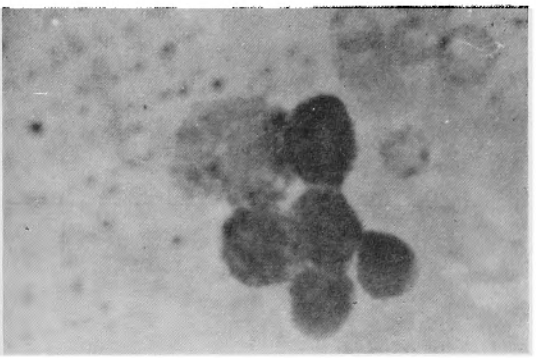

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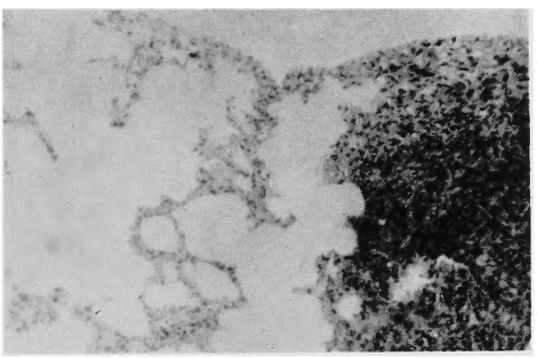

Fig. 27

EXPLANATION OF PLATES

Fig. 1 : Dividing cancer cell in the blood from a patient of gastric cancer, with large nucleus and slightly basophilic protoplasm. ×500

Fig. 2 : Cancer cell in the blood from a patient of gastric cancer with aberrant chromatin and 3 nucleoli. ×500

Fig. 3 : Cancer cell in the blood from a patient of gastric cancer, accompanied with an atypical cell. ×500

Fig. 4 : Cancer cell in the blood from a patient of gastric cancer. ×500

Fig. 5 : Cancer cell in the blood from a patient of ileocecal cancer, with large nucleolus nucleus ratio. ×500

Fig. 6 : Atypical cell in the blood from a patient of gastric cancer, with vacuolation in nucleus. ×500

Fig. 7 : Atypical cell in the blood from a patient of melanoblastoma. ×500

Fig. 8 : Atypical cell clump composed of about 30 cells found in the blood from a patient of gastric cancer. ×500

Fig. 9 : Atypical cell (megacaryocyte) observed in the blood from a patient of gastric cancer. ×500

Fig. 10 : Atypical cell with a large and dense nucleus observed in the blood from a patient of gastric cancer. ×500

Pig. 11 : Atypical cell of unknown origin, separated from the blood specimen of a patient of gastric cancer (PAS stain). ×500

Fig. 12 : Atypical cell in the blood from a patient of gastric cancer with a large nucleus and aberrant vacuoles. ×500

Fig. 13 : Artificial lung metastasis of MC/5, on the 26th day after intravenous injection of the cell suspension. ×200

Fig. 14 : Bashford carcinoma cells and cell debris in the coarse cell suspension. ×500

Fig. 15 . Artificial lung metastasis of Bashford carcinoma on the 31st day after intravenous injection of the cell suspension.

Fig. 16 : Bashford carcinoma cell still floating in the peripheral blood 30 minutes after intravenous injection of cell suspension. ×500

Fig. 17 : Lung, 2 hours after intravenous injection of Bashford carcinoma cell suspension. Polymorphonuclear leucocytes around the tumor cells (arrow). ×200

Fig. 18 : Lung, 6 hours after intravenous injection of Bashford carcinoma cell suspension. Tumor cell (arrow) permeating the lung capillary (double arrow) which is filled with leucocytes and erythrocytes. ×200

Fig. 19 : Lung, 12 hours after intravenous injection of Bashford carcinoma cell suspension. Cancer cell proliferation (arrow) in the perivascular space. ×50

Fig. 20 : Lung on the 11th day after injection of Bashford carcinoma cell suspension. Massive and infiltrative growth of the tumor. ×50

Fig. 21 : Lung on the 31st day after injection. The same findings as seen in Fig. 20. ×50

Fig. 22 : Lung on the 31st day after injection (Weigert stain). ×50

Fig. 23 : Metastasis of Bashford carcinoma in the heart, on the 16th day after injection. ×50

Fig. 24 : The same as Fig. 23 (magnified). Cancer cells invading into myocardial layer. ×200

Fig. 25 : Bashford carcinoma cell observed in the intracardiac blood of the 8th day after intravenous inoculation of Bashford carcinoma cell suspension. ×500

Fig. 26 : Cell clump composed of Bashford carcinoma cells in the intracardiac blood of the 14th day after intravenous inoculation of the carcinoma cell suspension. ×500

Fig. 27 : Lung with metastastic focus caused by transfusion of intracardiac blood of the 8th day after intravenous inoculation of Bashford carcinoma cell suspension. ×50